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FROM GENETIC ASSOCIATIONS TO BIOLOGICAL IMPLICATIONS IN MULTIPLE SCLEROSIS

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ABSTRACT

Every year, 600 people in Sweden develop MS, making it the second most common cause of disability (after accidents) in young adults. The pathophysiology is characterized by inflammation of the central nervous system and impaired neuronal signaling. Although the cause of MS remains elusive, important environmental and genetic contributors to disease risk have been identified. In order to develop better treatment strategies these risk modifying elements need to be functionally understood in the context of MS. Since the vast majority of currently known genetic and environmental factors increasing MS susceptibility have only been discovered in the past 5 years, now is the time to elucidate their biological foundation. This thesis, as the title suggests, focuses on the genetics of MS and how it impacts pathology. In time and content, it straddles the shift between searching for genetic associations (paper I), and understanding their clinical and biological implications (papers II-IV).

The main scientific objective of my PhD project was to characterize the association between the gene encoding the IL-7 receptor α-chain (*IL7R*) and MS susceptibility. First, we show that *IL7R* genotype does not impact clinical characteristics of MS such as disease severity or age at onset (paper II). This suggests that the link to MS susceptibility is indeed due *IL7R*'s influence on disease triggering events rather than an effect of altered clinical manifestation. In paper III we confirm previous reports that the MS associated allele (*IL7*R*C) causes increased expression and production of an alternatively spliced, soluble receptor isoform (sIL7Rα). We show that this isoform has intermediate affinity for IL-7, but contrary to membrane bound IL7Rα, does not bind TSLP. Despite competing with cell associated IL7Rα, sIL7Rα prolongs and potentiates IL-7's bioactivity both *in vitro* and *in vivo* by limiting excessive IL-7 consumption. Furthermore, MS patients homozygous for *IL7R**C have a 2-fold increase in plasma IL-7 levels, consistent with decreased IL-7 consumption as a result of higher sIL7Rα. In order to further map the interface between MS and IL-7 we went on to screen patients' serum IL-7 levels under different treatment regimens (paper IV). We found that MS patients receiving IFNβ therapy have increased serum IL-7 levels compared to untreated patients and healthy controls. The elevated IL-7 levels are coupled with both lower peripheral blood lymphocyte counts during IFNβ treatment, and reduced IL7Rα expression on those lymphocytes. Considering the stable rate at which IL-7 is typically produced, and our data supporting reduced IL-7 consumption, the increase in serum IL-7 is likely a product of slower depletion rather than increased production. Since IL-7 is an immune stimulatory cytokine associated with several autoimmune diseases, therapeutic modulation of this axis may improve clinical outcomes of MS patients, particularly for those receiving IFNβ treatment.

LIST OF PUBLICATIONS

I. Aulchenko YS, Hoppenbrouwers IA, Ramagopalan SV, Broer L, Jafari N, Hillert J, Link J, **Lundström W**, Greiner E, Dessa Sadovnick A, Goossens D, Van Broeckhoven C, Del-Favero J, Ebers GC, Oostra BA, van Duijn CM, Hintzen RQ; Genetic variation in the KIF1B locus influences susceptibility to multiple sclerosis. *Nat Genet.* 2008 Dec; **40(12):1402-3**.

II. **Lundström W**, Greiner E, Lundmark F, Westerlind H, Smestad C, Lorentzen AR, Kockum I, Link J, Brynedal B, Celius EG, Harbo HF, Masterman T, Hillert J; No influence on disease progression of non-HLA susceptibility genes in MS. *J Neuroimmunol*. 2011 Aug; **237(1-2):98-100**.

III. **Lundström W**, Highfill S, Walsh S, Beq S, Morse E, Kockum I, Alfredsson L, Olsson T, Hillert J, Mackall C., Soluble IL7Rα Potentiates IL-7 Bioactivity and Promotes Autoimmunity. *Manuscript*

IV. **Lundström W**, Hermanrud C, Sjöstrand M, Brauner S, Wahren-Herlenius M, Olsson T, Karrenbauer V, Hillert J, Fogdell-Hahn A; Interferon-β Treatment of Multiple Sclerosis Increases Serum Interleukin-7. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
BBB	Blood-Brain Barrier
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CIS	Clinically Isolated Syndrome
CD	Cluster of Differentiation
DNA	Deoxyribonucleic Acid
EIMS	Environmental Investigation of MS
ELISA	Enzyme-Linked Immunosorbent Assay
EBV	Epstein-Barr Virus
EDSS	Expanded Disability Status Scale
EAE	Experimental Autoimmune Encephalomyelitis
FACS	Fluorescence Activated Cell Sorting
GWAS	Genome Wide Association Study
HHV-6	Human Herpes Virus 6
HLA	Human Leukocyte Antigen
IFN	Interferon
IL	Interleukin
KI	Karolinska Institutet
MRI	Magnetic Resonance Imaging
MHC	Major Histocompatibility Complex
MS	Multiple Sclerosis
MBP	Myelin Basic Protein
MOG	Myelin Oligodendrocyte Protein
NK cell	Natural Killer Cell
NIH	National Institutes of Health
PCR	Polymerase Chain Reaction
PPMS	Primary Progressive Multiple Sclerosis
qPCR	quantitative real-time PCR
Treg	Regulatory T-cell
RRMS	Relapsing Remitting Multiple Sclerosis
RNA	Ribonucleic Acid
SPMS	Secondary Progressive Multiple Sclerosis
STAT	Signal transducer and activator of transcription
StopMS	Stockholm prospective study of Multiple Sclerosis
SNP	Single Nucleotide Polymorphism
TSLP	Thymic Stromal Lymphopoietin

1 INTRODUCTION

1.1 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic, heterogeneous disease of the central nervous system (CNS), characterized by local inflammation and myelin destruction in the brain and spinal cord. The name *multiple sclerosis* refers to the characteristic sclerotic plaques, which appear as a result of local inflammation in the CNS. This inflammation is mainly due to leukocyte infiltration of the brain and spinal cord, and leads to targeted destruction of the myelin sheath (*1*). CNS myelin is made up of cellular membrane from oligodendrocytes and facilitates neuronal communication through axons by providing insulation. This insulation ensures rapid and protected carriage of action potentials through the axon. As the disease progresses and damage to the oligodendrocytes increases, demyelination of neuronal axons worsens and cutting of axons and neuronal atrophy occur with increasing frequency (*2*). The symptoms of MS can vary widely based on what part of the brain is affected by demyelination. Typically the initial phase is characterized by a relapsing-remitting disease course where bouts of worsened disability and recovery follow each other (RRMS). Ultimately, a progressive accumulation of neurological disability ensues (secondary progressive MS - SPMS). The remaining group (10-15% of patients) has a primary progressive, bout-free disease course and typically a later disease onset (PPMS; Figure 1).

A person's first MS-like neurological symptom is usually referred to as a Clinically Isolated Syndrome (CIS). For an MS diagnosis to be made according to current standards (McDonald criteria) a second event, separate in time or space is required (*3*). A recent report looking at the risk of developing chronicity and hence MS within 20 years of presenting with CIS was 63% (*4*). The risk of MS went up to 82% if the CIS was accompanied by abnormal magnetic resonance imaging (MRI) findings. Although a clear majority of CIS cases eventually go on to develop MS, these reports (*4-6*) highlight the fact that not everyone undergoing an MS-like CIS end up with an MS diagnosis (Figure 1). Whether clinically indistinguishable pathophysiological differences between CIS events determine who develops MS and who does not remains to be determined. An alternative possibility is that the chronicity of MS is established by separate mechanisms than those triggering the initial event of neuro-inflammation, i.e. physiologically identical CIS events could lead to different outcomes. If this is the case, it indicates that the initial loss of self-tolerance is controlled by different mechanisms than the formation of a stable myelin-reactive immunological memory.

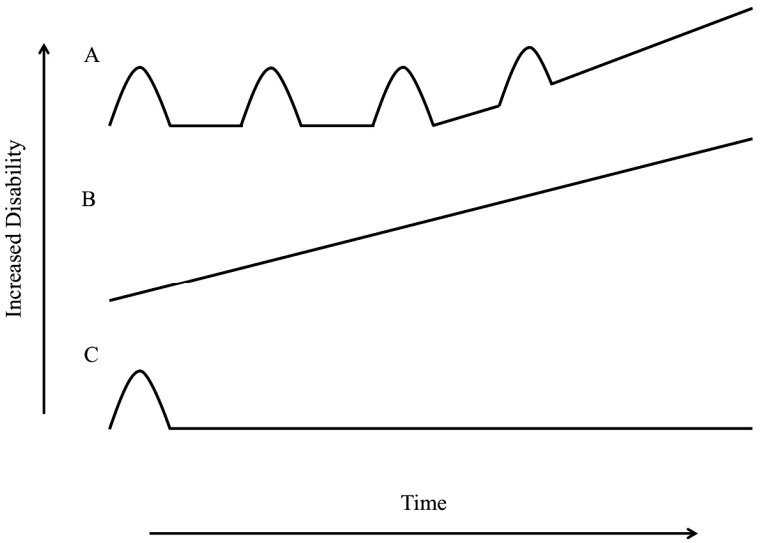

Figure 1: Common disease phenotypes of MS and CIS. (A) In most cases, a CIS later develops into relapsing-remitting disease which with time develops into secondary progressive MS. (B) 10-15% of MS patients present with a bout free phenotype called primary progressive MS. (C) 20-40% of CIS cases never develop MS.

The average age at RRMS onset is around 30 years. Life expectancy is not severely impacted by MS, but patients do have a slight increase in mortality (*7*). This increase is primarily accounted for by "death due to MS" rather than increased death due to co-morbidities. Cancer for example seems convincingly less likely to be the cause of death of an MS patient compared to the general population, perhaps due to increased immune reactivity in some MS patients (*7, 8*). MS is the most common neurodegenerative disorder in Sweden with a prevalence of around 1 in 520. Women are over two times more likely (prevalence in Sweden ≈ 1 in 380) than men (1 in 880) to develop disease (*9*). Although longevity is not severely affected, MS poses a huge burden both to the affected individual and his or her family-members in terms of reduced quality of life, as well as to society due to the high cost of treatment (*10*).

1.1.1 Who Gets MS?

The first evidence of increased familial recurrence of MS was reported over 100 years ago (*11*). Since then, it has been shown through numerous epidemiological studies that MS is a complex disease with both environmental and genetic cues affecting susceptibility. For example, concordance rate among monozygotic twins is around 25%, whereas it is approximately 3% for dizygotic twins (*12, 13*). This discrepancy based on genetic sharing shows that inherited genotype is important, but cannot by itself explain why some people develop MS. Thus, additionally to providing a genetic base for susceptibility, these studies tell us that environmental factors also play an important role in determining risk of MS (Figure 2).

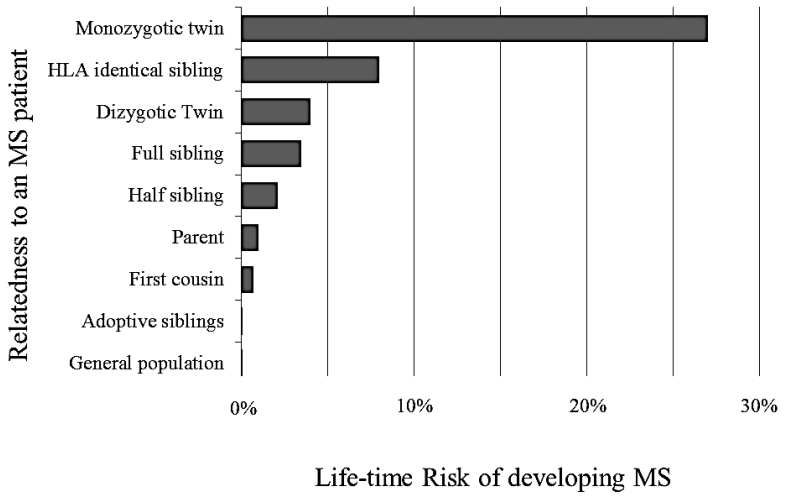

Figure 2: Life time risk of developing MS is based on relatedness to another person with MS. The closer related (higher genetic sharing) a proband and a relative are, the higher that relative's lifetime MS risk. The graph is based on a meta-analysis of familial MS studies by Compston et al (*1*).

1.1.1.1 Genetics

Many linkage studies looking at family pedigrees with high MS prevalence have tried to identify rare mutations capable of causing MS independently of environmental influence or conversely, environmental triggers strong enough to cause disease without genetic predisposition, but with little luck. Instead the new era of genome wide association studies (GWAS) has provided an approach which has been much more fruitful. The number of genetic regions that are convincingly associated with disease risk has increased dramatically from 1 to more than 50 in the last 6 years (*14*). Interestingly, most of the disease associated genes regulate immune rather than neurological functions. This fact underlines the importance of immune pathways in the initial break of self-tolerance causing disease onset. Disease progression and severity however do not seem to be regulated by the same genetic polymorphisms that predispose to acquiring disease. The alleles associated with MS susceptibility have shown a lack of impact on MS severity in several studies including *Paper II* of this thesis (*15-17*). Recent multi-center GWAS looking for markers of disease severity paint a complex and heterogeneous picture of genetic influence, with several of the strongest candidates being genes regulating neuronal function rather than general immunity (*18, 19*). Together these studies suggest that MS onset is triggered by general immune mechanisms, whereas different, organ specific modulators guide disease progression. In support of that model, several of the genes associated to MS susceptibility have also been associated to other autoimmune disorders e.g. psoriasis (*IL12B*), Crohn's disease (*STAT3*), rheumatoid arthritis (*IL2RA*) and type 1 diabetes (*IL7R*) (*14*). Given these overlaps between diseases, it seems likely that these polymorphisms affect the initial break of self-tolerance (in a disease unspecific manner)

through common immunological mechanisms, rather than later events in already established disease.

The strongest genetic associations to MS (and indeed most other autoimmune diseases) lie in the Human Leukocyte Antigen (HLA) region. Whereas risk alleles of the genes found in GWAS have odds ratios of about 1.2, the strongest HLA association (HLA*DRB1501) has a reported odds ratio of ~3 (>20 for HLA*DRB1501 homozygotes lacking HLA*A02 (*20*)). The HLA association strengthens the case that immunological processes including antigen presentation are at the core of determining MS risk.

1.1.1.2 Environment

Environmental triggers of MS are less well characterized but known to be important. The fact that monozygotic twin concordance (mentioned above) is ~25% and not 100% indicates environmental exposures have great influence on disease susceptibility. Furthermore, migration studies have shown that people moving from a low-prevalence area to a high-prevalence area tend to retain their low MS susceptibility whereas people moving the opposite direction i.e. from high to low prevalence areas reduce their risk of developing MS (*21*). This combination of epidemiologic data suggests that environmental exposures during childhood and adolescence are of essential importance for disease risk. Indeed, the positive effects of migrating from a high risk to a low risk area are greatest if the migration happens before the age of 15 (*22*). There are currently three environmental factors that are widely accepted to increase MS susceptibility: low vitamin D levels, cigarette smoking and Epstein-Barr virus (EBV) infection. Each is discussed below.

1.1.1.2.1 Vitamin D

One quite solid determinant in whether an area has low or high MS risk is latitude. It seems that the further an area is from the equator the higher the MS prevalence. This fact has pointed to ultraviolet radiation or vitamin-D as likely protective agents against MS development (*23*). Geneticists have tended to point out minorities with low MS incidence living near the poles e.g. the Sami of Scandinavia, as proof that the latitude gradient has more to do with ethnicity than sun exposure. Since environmental factors (such as sun-light exposure) are considerably harder to accurately quantify than genetic factors it remains challenging to accurately and completely understand the latitude effect. A recent study on American army veterans who served during the first gulf war surprisingly pointed to an increased MS incidence amongst African-Americans compared to Caucasian Americans (*24*). This is surprising considering MS rates in western Africa, where most African Americans stem from are much lower than in Europe, which is the main origin of Caucasian Americans. One clue to explaining this difference could lie in skin color. Higher skin pigmentation (i.e. darker skin) has been associated with decreased cutaneous synthesis of Vitamin D (*25*), and African American women have lower Vitamin D levels than Caucasian American women (*26*).

A link between Vitamin D insufficiency and several other immune mediated diseases has been observed, and is believed to be connected to effects on the adaptive immune response (*27*).

1.1.1.2.2 Epstein - Barr Virus

Infection with EBV has long been suspected of increasing MS risk. EBV infection is typically asymptomatic during childhood, but generally leads to infectious mononucleosis in adolescents or adults. The chance (or risk) of avoiding childhood infection is greatest in countries with high standards of living, hence the epidemiological map of infectious mononucleosis (implying a person was not exposed to EBV during childhood) correlates well with MS prevalence. Furthermore, the risk of several autoimmune diseases (as well as MS) in developed countries has steadily increased over the last few decades whereas the risk of infections has declined (*28*). One possible explanation for this phenomena is the so called "hygiene hypothesis" which states that a lack of exposure to common pathogens causes an increased risk of allergy and autoimmunity. This would explain the link between MS and infectious mononucleosis without necessarily providing a direct link between the two. A direct link is instead provided by the knowledge that people who remain uninfected with EBV throughout life have an extremely low risk of developing MS, whereas infectious mononucleosis increases the MS risk 2.3 fold compared to infection during childhood (*29, 30*).

1.1.1.2.3 Cigarette Smoking

There is clear evidence of an association between cigarette smoking and increased MS susceptibility (*31-34*). Swedish snuff, which is high on nicotine does not seem to have the same effect (*34*). It has been suggested that the increased MS risk amongst smokers (~50% between ever-smokers and never-smokers) combined with changing gender habits (more women smoke cigarettes today than previously) could provide the entire explanation as to why the gender gap between female and male MS incidence is growing (*35*). Several plausible explanations to the mechanism linking cigarette smoke and MS have been put forward including disruption of the BBB, increased nitric oxide production and immunomodulatory effects (*35*). Further investigation is needed to elucidate the exact biology of these intriguing findings.

1.1.2 MS Severity

MS severity is a very important, but not entirely straightforward concept. It is important since clinical trials and biological understanding of the disease rely on quantifying the level of disability and determine what affects it. Disability is defined by the World Health Organization as: "an umbrella term of impairments, activity limitations and participation restrictions" (*36*). The concept of disability is thus centered on a person's ability to interact with its environment, an ability strongly impaired by MS.

The most widely used scale for assessing MS disability is the Expanded Disability Status Scale (EDSS) outlined by Dr John Kurtzke in 1983 (*37*). Essentially, an EDSS score is determined based on disability in functional systems (pyramidal, cerebellar, brainstem, sensory, bowel/bladder, visual and cerebral) at lower scores, and mostly on motoric impairment at higher scores. The scale runs between 0 (no neurological symptoms) and 10 (death due to MS). The EDSS has received much criticism for various shortcomings including bad correlation with MRI lesions (*38*), poor consistency between ratings and raters (*39, 40*), too much focus on walking capacity and too little focus on cognitive impairment (*41*). Despite all these negative side notes, EDSS remains the most widely used disability scale in MS, possibly due to lack of competition (in quality not quantity). Furthermore, MS does not have many solid endpoints to tie a scale too. Death is a poor endpoint since MS does not have a very strong effect on longevity. Relapse-rate has also been proven to correlate weakly with overall disability, and is prone to variation between physicians' definition and calling of a relapse.

1.1.3 The Pathophysiology of MS

There is some controversy about whether the primary mechanism of MS is inflammatory or neurodegenerative (*42*). Most of the community remains convinced however that immune mediated inflammation plays a central role in pathogenesis credit to animal experiments and the efficiency of immune modulatory drugs. The onset of inflammation in MS is dependent on auto reactive, myelin specific lymphocytes entering the CNS. An obstacle for them to do so is the BBB, which provides the CNS with an immunologically protected environment. Previously the BBB was thought to disable lymphocyte trafficking into the cerebrospinal fluid (CSF) and the brain, and thereby rendering the CNS an immune privileged site. Instead, it seems that activated T-cells (the main leukocyte in CSF), by expressing adhesion molecules, integrins (including the α4-integrin targeted by the MS therapy natalizumab, discussed in chapter 1.1.4.2 (*43*)) and chemokine receptors, can actively diffuse across the BBB (*44*). In order for this to happen, T-cells specific for myelin antigens need to be activated in the periphery, leading to subsequent migration across the BBB. In the CNS, antigen-presenting cells (APCs, primarily activated microglia) reactivate the T-cells, which in turn induce specific degradation of the myelin sheath. It is unclear how the auto reactive, myelin specific T-cells are activated in the periphery, considering there is no lymph in the brain. CSF has the ability to drain antigens and immune cells to the cervical lymph nodes, and it has been suggested that it can substitute for the lack of a lymphatic system in the CNS and thereby present myelin antigens to auto reactive T-cells (*45-47*). Epidemiologic studies however have not been able to clearly establish an MS protective effect of childhood tonsillectomy (*48, 49*). Another hypothesis is that myelin proteins from damaged peripheral nerves provide the antigen needed for the APC triggered initial peripheral activation of T-cells (*50*). After activation these cells could cross the BBB and cause the more organ specific damage. This model works well to explain why immunization with peptides from myelin basic protein (MBP, a protein present in both central (Oligodendrocyte created) and peripheral (Schwann cell created)

myelin) triggers the MS animal model Experimental autoimmune encephalomyelitis (EAE) in mice. EAE resembles MS pathogenesis and can have a relapsing-remitting or progressive disease course depending on the specific model animal used. Finally, the concept of molecular mimicry has also gained much attention as a plausible explanation for peripheral activation of T-cells (*51*). The molecular mimicry hypothesis is based on the capacity of pathogen specific T-cell receptors (TCR) to cross-react with myelin peptides. For example, strong similarities in a peptide sequence from human herpesvirus 6 (HHV-6) and a different peptide from MBP have been shown to be similar enough that the HHV-6 peptide could be used to activate MBP specific T-cells from MS patients *in vitro* (*52*).

The main function of the immune system is to provide self-tolerance while maintaining a potent defense against harmful pathogens. This involves selection against auto-reactivity through maintenance of central tolerance (in the thymus) and peripheral tolerance. Both MS patients and healthy subjects have myelin specific naïve T-cells in their periphery, which have escaped central tolerance (*53*). Peripheral tolerance is a complex mechanism maintained by anergy, clonal deletion and regulatory T-cells (T-regs) to prevent an autoimmune response. Thus, for autoimmune events to happen in MS, myelin specific T-cells need to both escape central tolerance (which happens to everyone) and peripheral tolerance (which triggers the onset of myelin degradation). One issue with the break of peripheral tolerance is that presenting a myelin peptide on major histocompatibility complex (MHC) to a myelin specific T-cell is not enough. When an APC and a naïve T-cell interact, the T-cell will only become an activated effector if both signal 1 (antigen presentation on MHC) and signal 2 (co-stimulation, primarily by binding between CD28 on the T-cell and CD80/CD86 on the APC) is delivered by the APC. For this to happen the APC must itself be active, a process relying on binding of pattern-recognition receptors to pathogen (PAMPs) or tissue damage (DAMPs) derived antigens (*54*).

The strong genetic link to HLA class II as well as adoptive transfer experiments with myelin reactive T-cells in mice have led to the conclusion that MS is primarily a CD4+ (helper T-cell) mediated disease (*55*). Within the CD4+ family of T-cell subsets, initially type 1 T helper (Th1) cells were seen as likely drivers of inflammation. Much of the underlying work behind this hypothesis was carried out in the main animal model of MS: (EAE). Interleukin-12 is a critical factor for differentiation of naïve T-cells into Th1 cells and consists of two subunits: p35 and p40 (the complete heterodimer is sometimes referred to as IL-12p70). The work identifying Th1 cells as central in MS pathogenesis was largely based on studies of EAE in $p40^{-/-}$ mice (*56*) or with antibodies directed against p40 (*57-59*). However, a few years later another cytokine that also carries the p40 subunit, but in this case together with p19 was discovered: IL-23 (*60*). IL-23 was shown to be the critical cytokine rather than IL-12 in EAE pathogenesis (*61-63*), and the effect was shown to be Th1 independent but rather relying on a new IL-17 producing helper T-cell lineage (now known as Th17 cells) (*64*). IL-23 is essential for the maturation of Th17 cells and this cell subset is now receiving much attention for its involvement in EAE and MS. In a recent study, MS

patients during relapse were shown to have on average 3-fold higher Th17/Total CD4+ ratio in CSF compared to controls with other (non-inflammatory) neurological disorders (*65*). No difference was seen in peripheral blood.

Although Th17 is getting a lot of attention in MS research lately, it should be noted that there is considerable plasticity between T helper cell populations (*66*). For example, Th17 cells have been shown to start behaving like Th1s (produce interferon gamma) both *in vitro (67)* and in vivo (*68*) under inflammatory conditions. This gives some vindication to the early efforts identifying Th1 as the target cell subset.

1.1.4 MS Treatments

There are currently six approved disease-modifying compounds used for MS treatment. Additionally, autologous hematopoietic stem-cell transplantation after total body irradiation as a means of "resetting" the immune system in severe, progressive MS has shown promising results. A recent meta-analysis showed that the mean progress-free survival 3 years after treatment was 60-70%, impressive considering the clinical history of these cohorts (rapidly progressing disease) (*69*). The effects seem even better in RRMS subjects frequently showing improved EDSS scores. However, the treatment related mortality risk of approximately 2% is a serious obstacle to expand this treatment strategy amongst non-severe MS patients (*70, 71*).

1.1.4.1 Interferon Beta

IFNβ has been the most widely used therapy for RRMS treatment over the last 20 years. Although clinical trials have shown a 30% reduction in relapse rate and reduced MRI activity (*72, 73*), the long-term benefits in terms of reduced disability are disputed (*74, 75*). The mechanism behind IFNβ's clinical effects is not completely understood, but several possible explanations have been investigated. One hypothesis is based on IFNβ's immune modulatory properties as a skewer of the cytokine balance towards a more anti-inflammatory milieu (*76-78*). This "cooling" of the immune system may benefit MS patients by reducing the inflammation induced tissue damage of the CNS. Another hypothesis is based on IFNβ's ability to strengthen the integrity of the BBB by reducing expression of adhesion molecules needed for activated lymphocytes to migrate across it (*79*).

1.1.4.2 Natalizumab

The most effective treatment of RRMS to date is a monoclonal antibody called natalizumab. Natalizumab binds the alpha-4 integrin subunit (α4) which is a crucial component in the α4β1 and α4β7 integrins utilized by lymphocytes to migrate into tissue (including across the BBB into the CNS). The drug has been shown to reduce MRI identified lesions in the CNS as well as an overall reduction of disease progression (*43, 80*). One problematic side effect of disabling lymphocyte traffic across the BBB is the reduced ability of the immune system to deal with opportunistic infections.

Particularly the John Cunningham (JC) virus has been problematic, and led to serious side effects in the form of progressive multifocal leukoencephalopathy (PML) in RRMS patients on natalizumab treatment. As of May 2012, 212 cases of PML had been reported out of almost 100,000 RRMS patients treated with natalizumab (*81*). This serious side effect has led to natalizumab generally being prescribed only in severe MS cases, and after evaluation of other risk factors for PML including JC-antibody titers and previous administration of immunosuppressant drugs(*82*).

1.1.4.3 Glatiramer Acetate

Glatiramer acetate consists of a random peptide containing the four most common amino acids in MBP, and was originally intended to trigger EAE in mice. Instead it turned out to render rhesus monkeys immune to EAE induction (*83*), leading the investigators to research its potential as an MS drug. Its proposed mechanism of action is to induce tolerance against myelin antigens through competition with MBP peptides, thereby preventing an immune response against the myelin (*84*). Glatiramer acetate has similar long-term treatment efficacy for RRMS as IFNβ according to two recent studies (*85, 86*).

1.1.4.4 Other Treatments

Additionally there are three approved MS therapies prescribed more rarely than IFNβ, natalizumab and Glatiramer acetate. Except for Mitoxantrone, these are all used to treat RRMS exclusively.

- Fingolimod – The first oral RRMS drug Fingolimod modulates the sphingosine 1-phosphate receptor, which prevents lymphocytes from exiting lymph nodes. As a result, patients have ~50% reduced relapse rate and slowed progression of disability (*87*). Side effects of the induced immune modulation include opportunistic infections and skin cancer.

- Mitoxantrone – Used to treat both RRMS and SPMS, Mitoxantrone impairs DNA synthesis and repair leading to disabled lymphocyte proliferation. The drug is fairly well tolerated, although it should not be given for more than 2 years in total due to adverse side effects (*88*). It reduces relapse rate in RRMS and slows EDSS increase in both RRMS and SPMS.

- Teriflunomide – The latest approved treatment of RRMS, this oral drug blocks the synthesis of pyrimidine, thereby inhibiting DNA synthesis crucial for proliferating cells. The methodological reasoning is similar to that of Mitoxantrone i.e. inhibition of effector cells targeting myelin to undergo rapid proliferation and hence blocking powerful immune responses. Teriflunomide has similar effects on relapses and clinical outcomes as IFNβ and Glatiramere acetate with the added benefit of oral administration rather than injection (*89, 90*).

Furthermore, corticosteroids are sometimes given to RRMS patients as immune suppressants during a relapse. All the currently approved MS-therapies are in one way or another immune modulatory, and are focused on blocking crucial steps in immunity mediated myelin destruction (summarized in figure 3). Future challenges include developing therapies with organ specific effects such as stimulating re-myelination. Ongoing trials using autologous mesenchymal stem-cell transplantation in MS and neural stem-cell transplantation in amyotrophic lateral sclerosis could provide a first step in that direction (*91, 92*).

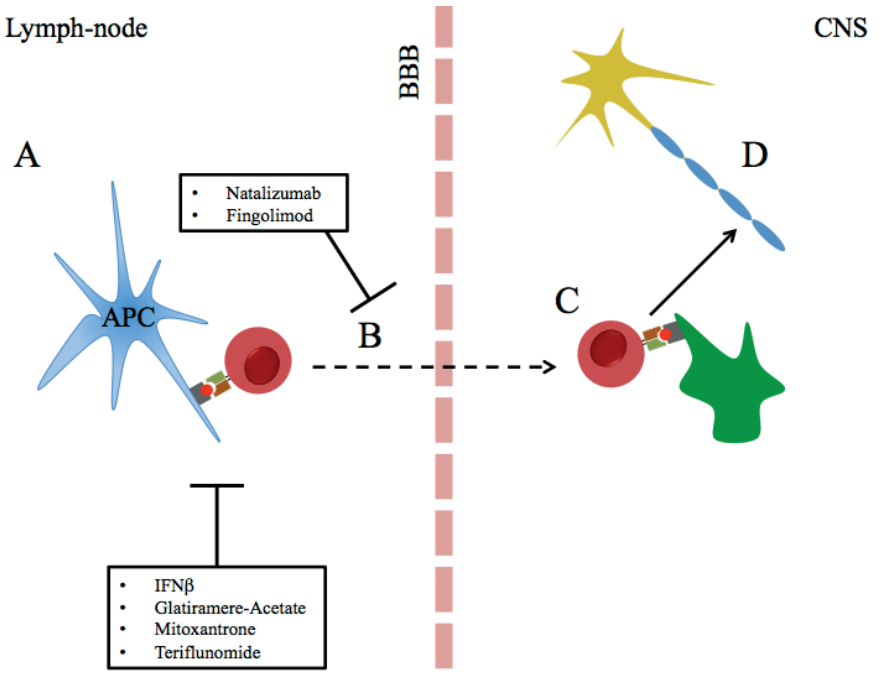

Figure 3: Schematic view of pathophysiologic targets of the currently approved MS drugs. APC (blue) presents a myelin peptide on MHC to an auto-reactive T-cell (red) leading to their activation and proliferation (A). These processes are targeted in different ways by the immune modulatory drugs IFNβ, Glatiramere acetate, Mitoxantrone and Teriflunomide. The activated T-cell by up-regulating integrins and chemokine receptors can then traffic out of the lymph node (inhibited by Fingolimod), into the blood stream and across the BBB (inhibited by Natalizumab) into the CNS (B). In the CNS, the T-cell is reactivated by local APCs (primarily activated microglia, green (C)) and induces an immune response against the myelin sheath (blue) covering a neuron's (yellow) axon (D).

1.1.4.5 Future Treatments

There are currently a large number of ongoing clinical trials of new therapies in MS. Most of these target different components of the immune system. A few of them are discussed below.

- Alemtuzumab – A monoclonal anti-CD52 antibody that leads to T-and B-cell depletion, but does not target hematopoietic stem cells. Alemtuzumab has showed great clinical efficiency when RRMS patients were treated early in disease (actually decreasing EDSS score 5 years into treatment) (*93, 94*). Relapse-rate and sustained disability is reduced by approximately 70% compared to IFNβ treatment.

- Daclizumab – Daclizumab is a monoclonal antibody targeting the IL-2 receptor α chain (CD25). CD25 is crucial for the autocrine IL-2 stimulation that T-cells utilize to induce proliferation upon antigen recognition. Combined treatment with IFNβ led to fewer new or enlarged MRI lesions but did not impact EDSS (*95*).

- Rituximab and Ocrelizumab – Anti-CD20 monoclonal antibodies targeting B-cells. B-cells are a logical target in MS since they both function as (myelin specific) antibody producers and professional APC (i.e. expresses MHC class II and hence have the ability to activate CD4+ T-cells). Rituximab and Ocrelizumab's efficacy is disputed, and side effects can be severe. Decreased MRI lesions have been reported (*96-98*), however it does not seem like these compounds will be new blockbuster drugs in MS.

- RTL1000 – Is an elegant approach to MS treatment. RTL1000 consists of a peptide which forms an MHC+myelin oligodendrocyte protein peptide (DR2+MOG_{35-55}) intended to deliver signal 1 in the absence of signal 2 to myelin specific T-cells. The intention is to induce peripheral tolerance through anergy of these myelin specific T-cell clones (*99*).

1.2 INTERLEUKIN-7

IL-7 was initially identified in as a growth factor for murine B-cell development (*100*). Later, it was established that IL-7 additionally promotes murine T-cell maturation (*101*) and that it exerts similar stimulatory effects on human lymphocytes. There is one big difference between IL-7's role in humans and mice however. The first report showing that people carrying *IL7R* (the gene encoding IL-7 receptor alpha chain (IL7RA)) loss of function mutations can develop severe combined immunodeficiency (SCID), surprisingly found that these patients although lacking T-cells had relatively normal B-cell numbers in blood (*102*). *IL7*$^{-/-}$ and *IL7R*$^{-/-}$ knockout mice on the other hand have severe T- and B-cell lymphopenia (*101, 103, 104*). Thus, as far as adaptive immunity goes, in humans IL-7 is mainly a regulator of T-cell development and survival whereas mice additionally need IL-7 to generate B-cells.

Apart from its non-redundant status in the development of adaptive immune cells, IL-7 has recently been identified as a key cytokine for innate lymphoid cells (ILC). Initially, ILCs were thought to exclusively consist of natural killer (NK) cells (*105*), but it is now known that the ILC repertoire encompasses a wide range of cell subsets with different immunological functions (including natural helper cells (NH) and lymphoid tissue inducer cells). Interestingly, all of these ILC subsets express IL7RA, and IL-7 stimulation is important for their development and survival (*106*).

1.2.1 IL-7 Production

IL-7, ironically, is not technically an interleukin. The name *interleukin* implies signaling between two leukocytes, and whilst the primary function of IL-7 signaling is to stimulate growth and survival of different leukocyte subsets, the main producers of this cytokine are stromal cells of the primary and secondary lymphoid organs (*107*). The production of IL-7 is generally considered constitutive i.e. it is produced at a stable rate. IL-7 concentration in blood is thus primarily regulated by consumption rather than production. Indeed, under lymphopenic conditions (i.e. few IL-7 consuming cells) IL-7 levels are elevated, and there is an inverse correlation between T-cell numbers and serum IL-7 levels (*108, 109*). The strength of the IL-7 signal on an individual lymphocyte is thus regulated by receptor expression rather than cytokine production.

1.2.2 IL-7 in the Thymus

In the thymus, IL-7 has a central role in the maturation of thymocytes to single positive T-cells. During this process called thymopoiesis, thymocytes are dependent on IL-7 stimulation at several stages (*110*). IL-7 is always present in the thymus since thymic stromal cells are its main producers. Signaling is instead tightly controlled by IL7RA up- and down regulation during the different stages of T-cell maturation. Simply put, IL7RA expression is absent in early thymic progenitor cells, present on double negative thymocytes (CD3+/CD4-/CD8-), down-regulated again during the double positive stage (when thymocytes undergo positive selection; CD3+/CD4+/CD8+) and then re-

expressed on single positive naïve CD4+ and CD8+ T-cells during negative selection and when exiting the thymus. Maintained surface IL7RA expression is crucial for T-cell's survival and for the potential of homeostatic proliferation in the peripheral lymphoid organs (*111*).

1.2.3 IL-7 in the Periphery

After puberty the thymus slowly starts shrinking and eventually loses most of its ability to provide the T-cell niche with naïve cells (*112*). Instead, peripheral maintenance and proliferation of T-cells becomes increasingly important. All naïve T-cells have low affinity for self-peptides presented on MHC as a result of positive and negative selection in the thymus. Survival of naïve T-cells utilizes this axis and is dependent on TCR stimulation through self-peptide + MHC complexes as well as anti-apoptotic signals delivered by IL-7 (*113*). Under normal physiological conditions, these signals primarily induce survival and cycling of peripheral T-cells, and the strength of the combined stimulation (self-peptide + MHC and IL-7) is not enough to induce activation and proliferation but mainly serves to prevent apoptosis. Under lymphopenic conditions however, homeostatic proliferation in the periphery occurs. This lymphopenia induced expansion of naïve and memory T-cells can be initiated by disease (e.g. HIV) or as a result of therapy (e.g. chemotherapy against various tumors). As IL-7 consumption is reduced, serum IL-7 levels rise and reach a threshold where cells start dividing. This homeostatic proliferation is less tightly controlled than thymic expansion, and is believed to be a contributing factor to the link between lymphopenia and autoimmunity (*114*). Memory T-cell homeostasis is also dependent on IL-7 signal, however they do not need TCR stimulation by self-peptide + MHC complexes to the same extent as naïve cells do (*115*).

There is mounting evidence that T-regs are less dependent on IL-7 stimulation than other T-cell subgroups. Firstly, IL7RA expression is low or absent on T-regs, and its expression inversely correlates with the expression of the forkead box P3 protein (FoxP3, a T-reg marker) and T-reg's suppressive capacity (*116*). In the periphery, T-regs are not dependent on IL-7 for survival or homeostatic proliferation (*117*). However, thymopoiesis seems to be IL-7 dependent also in the context of T-reg production (*118*). Together, this data suggests that although IL-7 signaling is non-redundant for all thymopoiesis, in the periphery, activation and survival signals mediated by IL-7 favor non T-reg subsets. Indeed, clinical trials with recombinant human IL-7 (rhIL7) have shown preferential expansion of non T-regs resulting in a diminished relative frequency of regulatory T-cells (*119, 120*). In contrast, recombinant human IL-2 therapy skews the T-cell repertoire in favor of T-regs (*121*).

1.2.4 The IL-7 Receptor

The IL-7 receptor is a heterodimer consisting of the IL7RA chain and the common gamma chain (γc or CD132). Both these chains form other cytokine receptors when complexing with different chains (figure 4). The sister cytokine of IL-7, Thymic

stromal lymphopoietin (TSLP) which utilizes IL7RA for signaling, can to some degree substitute for IL-7 signaling. This is thought to be the reason why *IL7*$^{-/-}$ mice are less T-cell lymphopenic than *IL7R*$^{-/-}$ mice (*122*). Shortly after the successful identification of IL-7 (*100*), the IL-7 receptor was cloned and characterized by Goodwin et al (*123*). Quite soon, IL7RA by itself was shown to have intermediate affinity for IL-7 (dissociation constant in the micro molar (μM) range) whereas the complex of IL7RA and CD132 binds IL-7 stronger (dissociation constant in the pico molar (pM) range; (*124*)). IL7RA expression is tightly regulated both during T-cell development and in mature T-cells. As discussed earlier (Section 1.2.1), IL-7 production is stable and hence the main tool for controlling signal strength is through regulation of IL7RA expression. This is actively carried out for example upon IL-7 stimulation when IL7RA is down-regulated. By regulating IL7RA expression, IL-7 is preserved for other cells in an altruistic fashion, which ensures that a limited resource (IL-7) is not excessively consumed (*110, 125*).

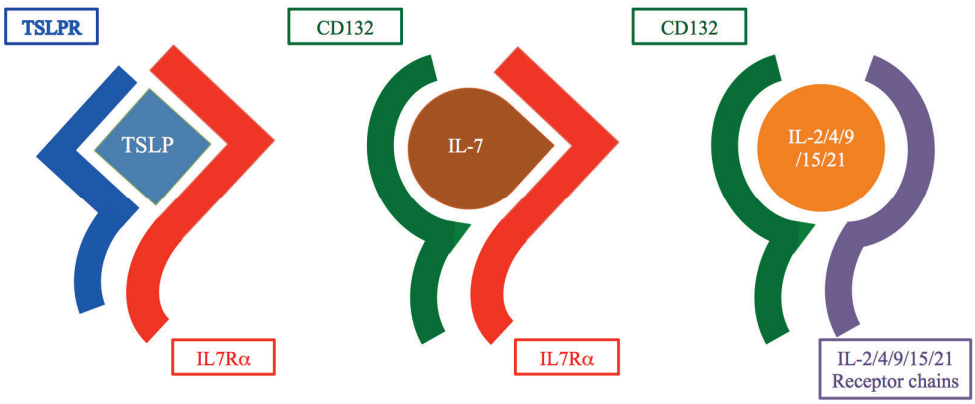

Figure 4: Both chains of the IL-7 receptor can form other cytokine receptors when bound to alternative receptor chains. IL7RA forms the TSLP receptor in complex with the TSLPR chain (left). CD132 can form six different cytokine receptors based on which additional receptor chain it complexes with (right).

1.2.5 IL-7 in Autoimmunity

Counter-intuitively, there is a well-established link between lymphopenia and autoimmunity (*126-128*). IL-7 is likely important in this chain of events due to the fact that its concentration in plasma inversely correlates with lymphocyte counts and IL-7 signaling induces homeostatic proliferation at high concentrations (*114, 129*). Furthermore, IL-7 has been shown to be a co-factor in the development of autoimmunity based on studies in animal models. IL-7 transgenic mice develop an autoimmune dermatitis phenotype, and administering IL-7 worsens mouse models of MS and ulcerative colitis, whereas blocking of IL7RA mitigates disease (*130-132*).

Further evidence of IL-7's involvement in autoimmune disease comes from the large number of autoimmune diseases genetically associated to polymorphisms in the *IL7R* gene (which encodes for IL7RA). The alleles associated with increased risks of MS (*133, 134*), ulcerative colitis (*135*), sarcoidosis (*136*) and primary biliary cirrhosis (*137*) are in strong linkage disequilibrium, and hence usually co-inherited. This fact suggests that these diseases share a common biological explanation to the genotype-autoimmunity link. Evidence put forward by others (*134, 138, 139*) as well as in paper III of this thesis support an involvement of soluble IL7RA. Additionally MS has been genetically associated with the IL-7 encoding gene *IL7 (14)*. This finding is relatively new and the mechanism behind is yet to be investigated, but it does provide the first cytokine + receptor pair convincingly associated to MS susceptibility.

1.3 SOLUBLE RECEPTORS

The biology of soluble receptors is a nascent field in immunology. Conservation across species and their abundance in serum indicates that these proteins have important biological functions (*140*). Soluble cytokine receptor's functions vary from reducing signaling strength (e.g. soluble IL1RII (*141*)) to greatly enhancing bioactivity of the target cytokine (e.g. soluble IL15Rα (*142, 143*)). Some soluble receptors have shown various abilities under different conditions. Soluble IL2Rα, has been reported to both potentiate (*144, 145*) and diminish (*146*) IL-2 signaling on T-cells. Similarly, a previous report on soluble IL7RA chain (sIL7RA) has shown IL-7 inhibition in the presence of an IL-7Rα-Fc fusion protein constructed to mimic sIL7RA (*147*). Our data, on the other hand show a preserving and potentiating effect of sIL7RA on IL-7 signaling in various experimental systems (paper III).

There are two methods utilized by cells in order to produce soluble receptor isoforms: membrane shedding and alternative spicing (*140*). Membrane shedding is based on proteolytic cleavage of the extracellular domain of membrane bound receptors (e.g. TNF receptor 2 (*148*)), whereas alternative splicing of mRNA (messenger ribonucleic acid; e.g. IL-9 receptor alpha chain (*149*)) leads to receptor secretion. Typically this is due to the alternative splicing generating a different mRNA molecule, which is then translated into a protein isoform lacking the membrane-anchoring domain (figure 5). Soluble IL7Rα is primarily generated through this alternative splicing based mechanism (*150*). It is present at high molar excess compared to IL-7 (>1000-fold, paper III), and its abundance is highly dependent on *IL7R* genotype.

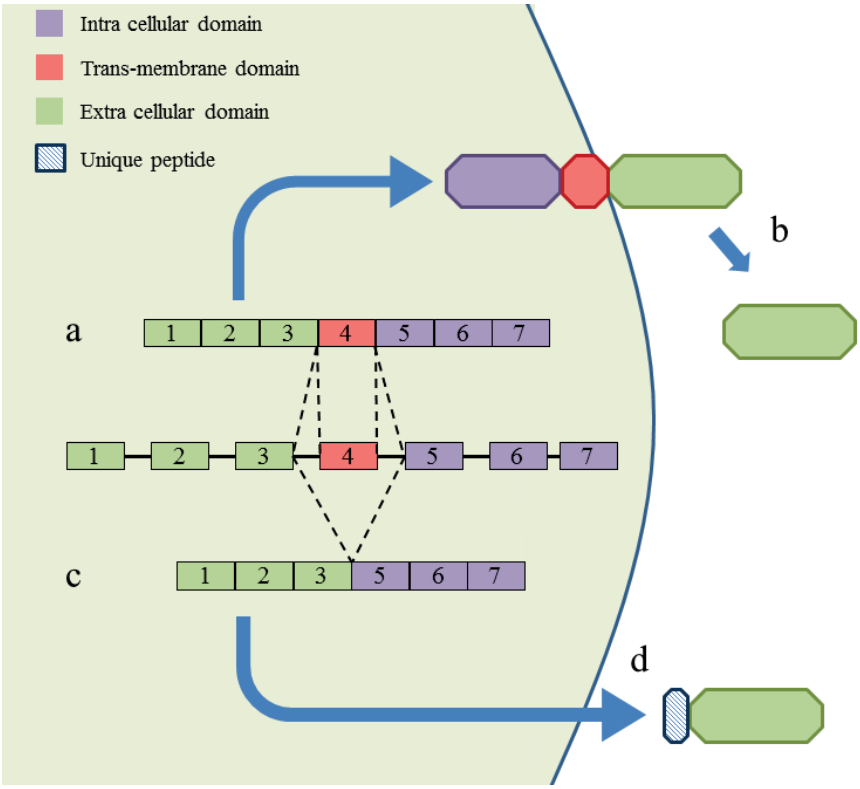

Figure 5: Generation of soluble cytokine receptors. (a) Intron splicing generates an mRNA molecule containing all exons (in this example exon 1-7). Translation renders a full-length protein containing intra-cellular, trans-membrane and extra-cellular domains. (b) The extra-cellular portion of the full-length receptor is shed, generating a soluble isoform identical to the extra-cellular domain of the membrane anchored isoform. (c) The second option occurs when alternative splicing results in the production of an mRNA isoform lacking the exon encoding the trans-membrane domain (in this example exon 4). (d) Due to excision of exon 4, translation of this mRNA leads to production of a protein lacking membrane-anchoring capability. An altered reading frame (the nucleotide bases in exon 4 were not evenly dividable by 3: the size of an amino acid coding codon) results in translation of a unique peptide downstream of the extra cellular domain (blue striped).

The effects of soluble receptors on our immune system are as diverse as the effects of the cytokines they bind. Many have shown to have strong influence on disease and health. For example, the auto inflammatory Tumor necrosis factor (TNF) receptor associated periodic syndrome (TRAPS), can be caused by inability to shed TNF receptor 1 (*151*). High soluble IL2Rα levels are correlated with worse outcome in Hodgkin's disease (*152*) and sIL7Rα is increased in HIV patients (*147, 150*). The future will hopefully bring greater understanding, and therapeutic approaches involving these mediators of cell-based signaling.

2 AIMS OF THESIS

The aim of this thesis was to determine how genetic risk factors, particularly the *IL7R* association, influence MS pathology. Specifically the core aims of each project are listed below.

Paper I: The genetic associations to MS susceptibility prior to this study where in genes regulating T-cell response and without clear organ specificity. This paper investigates a gene with documented importance for CNS function: *KIF1B,* which encodes the kinesin family member 1B protein.

Paper II: Several genetic associations to MS susceptibility had been made prior to this paper. Our aim was to evaluate whether polymorphisms associated with MS risk also impacted MS severity. An alternative hypothesis going in to this project was that some MS susceptibility markers were actually affecting severity and thus leading to quicker and more accurate diagnosis.

Paper III: The first non-HLA gene associated with MS susceptibility was the *IL7R* gene, which encodes the IL7RA chain. This paper elucidates the functional implications of *IL7R* genotype, and how it influences MS susceptibility. Specific attention was given to the biology of soluble IL7RA.

Paper IV: IFNβ is the most widely used first-line treatment in RRMS, and is known to have strong immune modulatory properties. We investigated how IFNβ administration impacts IL-7 biology.

3 MATERIALS AND METHODS

For detailed descriptions of methodology, see the individual methods section of that constituent paper. A brief overview of the methods used most follow in this chapter.

3.1 HUMAN SAMPLES

All patient and control materials used by me in these studies were collected after informed consent had been given by the individual donor. Ethical approvals were obtained from the regional ethical review board in Stockholm County. The protocols for enrollment of subjects were designed to cover several projects outside of this thesis, and thus are not specifically formulated for these experiments. Patients and controls were either enrolled in the EIMS (environmental factors in multiple sclerosis) or StopMS (Stockholm prospective assessment study of multiple sclerosis) projects.

3.2 IN VITRO EXPERIMENTS

3.2.1 Genotyping

All genotyping was performed by allelic discrimination on a Taqman platform (Applied Biosystems). The allele specific primers and probes (Assay ID for rs6897932 (paper III): C_2025977_10 and for rs10492972 (paper I): C_30400488_20) were ordered from Applied Biosystems.

3.2.2 Quantitative real-time PCR (qPCR)

Reverse transcription of complete mRNA was carried out with either random hexamers or oligo-dT primers. qPCR experiment setups differed between the different studies. In paper III, sIL7Ra and IL7Ra were quantified by comparison to plasmid standards of the relevant cDNA products with known concentration. GAPDH was used as house-keeping gene. In paper IV, relative quantity was determined between the product of interest and the house keeping gene HPRT1, without the use of a plasmid standard. Taqman quantification was carried out by primer+probe pairs ordered from applied biosystems (full-length IL7Ra: Hs00904814_m1, sIL7Ra: Hs00902337_m1, HPRT1: Hs01003267_m1 and GAPDH: Hs02758991_g1).

3.2.3 Enzyme linked immunosorbent assay (ELISA)

ELISAs were used for quantification of human IL-7 and sIL7Rα in serum and plasma. For IL-7 quantification a commercially available high-sensitivity kit was used (human IL-7 quantikine ELISA kit, R&D systems). sIL7Rα was quantified by a previously validated ELISA (*153*) using anti-sIL7Ra antibodies from R&D systems.

3.2.4 Cell culture

Cell cultures of PBMC were carried out in either serum-free (Aim-V, Life technologies) or complete (RPMI1640, Sigma-Aldrich supplemented with 10% heat inactivated fetal calf serum, penicillin, streptomycin and glutamate) media. Cell lines were cultured in Aim-V with supplemented IL-7 and split every two days. The rationale for using minimal media (serum free) for IL-7 studies was to ensure that bovine proteins did not interfere with cell signaling.

3.2.5 Flow cytometry

Flow cytometry experiments were done on a FACS Fortessa (BD biosciences) or a CyAn Dako (Beckman Coulter) machine. All cells were kept in cold PBS supplemented with 0.5% FCS and 0.1% NaN_3 during antibody staining and cell sorting. Intracellular staining was preceded by cell fixation and membrane permeabilization using BD Phosflow fix and perm buffers.

3.3 EAE

EAE is the most widely used animal model of MS, and has been an important tool for studying the biology and treatment of MS. Most approved MS therapies have been tested for treatment efficiency in rodent and primate EAE models (*154*). Although the clinical and histopathological symptoms of different EAE models correspond well with MS (there are relapsing remitting, monophasic and progressive EAE models), the triggering of disease differs (*155*). The most commonly used form of EAE (active-EAE) is induced by immunization with myelin peptides in complete freund's adjuvant (the method used in paper III of this thesis) (*156*). Alternatively myelin reactive T-cells can be adoptively transferred from an actively immunized mouse to induce "passive EAE" in a recipient animal (*157*). Hence, EAE results from auto-reactivity against a single antigen and is triggered by co-injection of an adjuvant, activating the innate immune system. MS on the other hand does not have a single antigen explaining its pathogenesis, and is not induced by administering adjuvant (*154*).

Our EAE experiments were based on immunization of C57BL/6 mice with MOG_{35-55} peptide and subsequent daily blinded scoring of disease symptoms. The EAE severity was determined as follows.

0. Normal
1. Flaccid tail, no paraparesis
2. Hind limb weakness evidenced by inability to right itself when placed on the back, or inability to grasp with its hindlimbs
3. Partial hind limb paralysis evidenced by inability to move one hind limb (e.g. to withdraw one limb when pinched, but able to bear weight on one limb
4. Complete hind limb paralysis evidenced by inability to move or withdraw hind limbs

5. Quadriplegia evidenced by inability to move front and hind limbs
6. Moribund or dead

Mice with an EAE score of 5 were sacrificed (humane endpoint).

The EAE model we used was monophasic i.e. animals developed a single MS bout. It would be interesting to see if a relapsing-remitting EAE model would have given different results.

4 RESULTS AND DISCUSSION

4.1 PAPER I

4.1.1 Our findings

We found an association between the rare C-allele of SNP rs10492972 and increased risk of developing MS (paper I (*158*)). The association was first seen in a small, inbred Dutch cohort, and later replicated in larger, outbred Dutch, Swedish and Canadian case-control materials. Rs10492972 is located in an intron of the *KIF1B* gene, which encodes the kinesin family member 1B (KIF1B). KIF1B is a member of the kinesin super-family of proteins which are all involved in axonal transport. Specifically, KIF1B is a "microtubule motor" which transports synaptic vesicle precursors from a neuron's cell body, through the axon to the synapse (*159*). Mutations in *KIF1B* can lead to the development of the peripherally demyelinating charcot-marie-tooth disease and *KIF1B*$^{-/-}$ mice die at birth of apnea due to impaired neuronal signaling (*160*). The finding that this gene impacted MS susceptibility was the first organ specific genetic association to MS-risk.

4.1.2 Other studies

Follow up studies of this study (*158*) have not seen the same association between MS and rs10492972*C (*161-163*). The difference in results is puzzling to say the least. Especially the results from a multi-center study by Booth et al (*161*), which included a different Swedish case-control material than the one used in our study, but did not pick up the same signal suggests the initial finding was a false positive. This is remarkable for several reasons, and needs to be explained further. Some possible explanations are listed below.

1. Our study generated a false positive due to genotyping errors. This hypothesis is not very likely considering the genotyping was carried out at three different locations.

2. Our study picked up a genotype effect unique to the cohorts studied. This would have been a likely discrepancy between ours and other studies if our patient and/or control cohorts were particularly homogenous. In such a case, a gene-gene or gene-environment interaction might have been unique to our study population. However, this also seems unlikely regarding the fact that our patient cohort came from three different countries.

3. The control groups differed. Perhaps differing criteria were used in picking control subjects.

4. The patient groups differed. An interesting side-note in a recent Russian study was that rs10492972*C was associated with increased RRMS risk but not PPMS risk (*162*), and a study on only PPMS came out negative (*163*). Differences in MS sub-phenotype ratios could provide a plausible explanation, especially since these ratios usually vary between different study centers. Further support for this hypothesis comes from the fact that the Australian case-control material, which gives the lowest odds ratio for rs10492972*C in the follow up study (Figure 6) was enriched for PPMS (ANZgene cohort in (*164*); >25% PPMS versus ~10% PPMS in general MS population).

5. We made a chance finding. P-values are calculated based on numerous assumptions about the investigated cohorts, and reflect an estimated risk of a positive finding being a false positive. Although the p-values in both these studies look impressive, there is a risk they are somewhat deflated, and hence there is still a non-negligible risk that our finding simply was a false positive by chance.

6. Since the genotyping in our study happened in three different places, I only have complete insight into the Swedish cohort's experiments and results. Perhaps something went wrong in the interpretation or generation of the other groups' data.

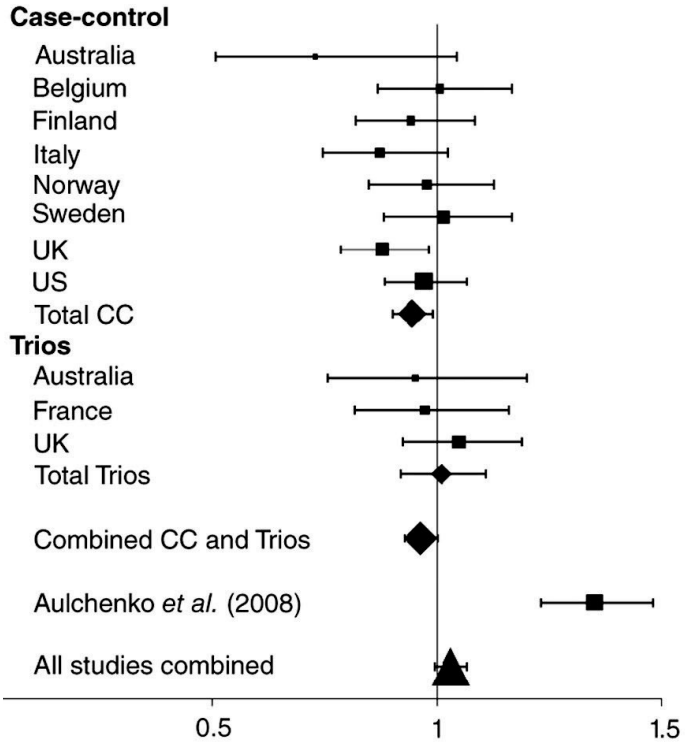

Figure 6: Odds ratios for the association between rs10492972*C carrier-ship and MS susceptibility. Reprinted by permission from Macmillan Publishers Ltd: Nature Genetics (*161*), copyright 2010.

It is important to clarify why the different studies on rs10492972 have yielded so different results. Two of the reports were multi-center studies using stringent statistical analysis and yet they reach completely different conclusions (*158, 161*). Both these studies included Swedish cohorts of patients and controls. Surprisingly, whereas the Swedish cohort used for paper I showed a statistically significant rs10492972 impact on MS risk, the cohort used in the follow-up study did not. It would be interesting to see if the odds ratios are similar in these materials regardless of MS sub-phenotype, particularly RRMS vs PPMS.

4.2 PAPER II

A genetic association to MS susceptibility could just as well be a modulator of clinical characteristics causing earlier diagnosis. For example, if a patient group and a control group are age matched, a genetic predisposition leading to an earlier age at disease onset would be indistinguishable from a susceptibility factor. Likewise, genes influencing the speed of disease progression could lead to a clearer phenotype, and hence quicker diagnosis. In this study we evaluated the impact of 5 SNPs (Table 1) known to impact MS susceptibility on clinical parameters. The objective of this project was to elucidate if any of these were surrogate markers for increased disease severity or earlier age at onset. Our patient cohort consisted of Swedish and Norwegian MS patients of Scandinavian origin.

Gene	SNP
IL7R	Rs6897932
IL2RA	Rs2104286
CLEC16A	Rs3184504
CD226	Rs763361
SH2B3	Rs3184504

Table 1: The SNPs included in paper II and their genetic context

We evaluated how these 5 genotypes influenced age at onset, MS severity score and time to an EDSS score of 6. None of the SNP alleles influenced these clinical parameters. Furthermore, we separated MS patients based on the number of these risk alleles they carried. No clinical difference based on number of genetic predispositions was seen. Other groups have also reported this phenomenon: susceptibility linked alleles not influencing disease severity (*16, 17, 165*). Whether effects exist, that are not detected with current methodology will have to be explored further.

We were disappointed at first that we did not discover any significant associations between these markers and clinical parameters. However, negative data, when gathered from such a large patient material as this one (1776 patients) can be highly relevant for increased understanding of disease mechanisms. As discussed previously (Section 1.1.1.1) it seems with our current knowledge as though the risk of MS onset and the clinical course of MS are dictated by different mechanisms. The results from paper II support this model. In terms of our further ambitions of dissecting the mechanistic explanation for the *IL7R* association (paper III), it was important to confirm that *IL7R**C actually worsens MS *risk* rather than MS course, at least according to the outcome measures we looked at here.

4.3 PAPER III

In 2007, the IL7Rα encoding gene *IL7R* was confirmed as the first new gene associated to MS susceptibility in over 30 years (*133, 134, 166*). It was the first non-HLA and last pre-GWAS genetic association found. The effect is attributable to a functional SNP (rs6897932) in the region encoding exon 6 of IL7RA. Exon 6 is the trans-membrane domain of IL7RA, and several reports including paper III of this thesis have linked *IL7R* genotype to the degree of exon 6 inclusion in the transcribed protein (*134, 167, 168*). The MS predisposing genotype (Carrying a cytosine at rs6897932, hereafter referred to as *IL7R**C) is associated with increased skipping of exon 6, resulting in higher production of a soluble IL7RA isoform (sIL7RA; Figure 7b). The rationale for this study was to determine how the *IL7R**C (and indirectly sIL7RA) contributed to increased MS risk.

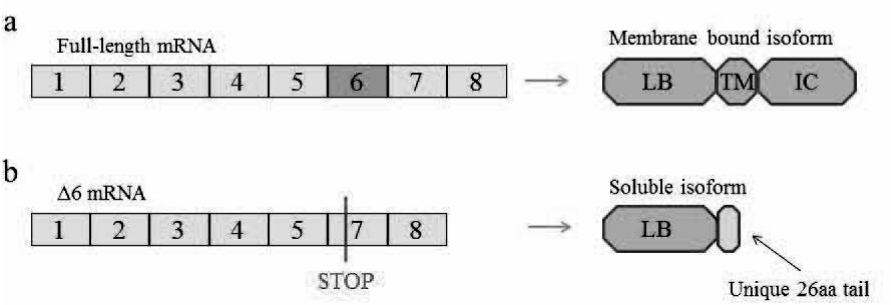

Figure 7: Alternative splicing of IL7Rα. Full-length mRNA is translated into the full-length protein containing a ligand binding (LB), trans-membrane (TM) and an intra-cellular domain (IC; a). Splicing out exon 6 generates an mRNA molecule with a shifted reading-frame downstream of exon 6 and a premature stop-codon. Translation produces a soluble IL7Rα isoform lacking TM and IC domains, but carrying a unique 26 amino acid peptide tail at its C-terminus end (b).

4.3.1 sIL7RA binds IL-7 but not TSLP

We used a human embryonic kidney cell-line (HEK293E) to produce sIL7Rα. It displayed intermediate binding affinity to IL-7 (nanomolar (nM) dissociation constant (K_d)), but in contrast no affinity for the other known IL7Rα signaling cytokine TSLP as measured by surface plasmon resonance (SPR; Figure 8). This indicates that sIL7Rα probably only affects IL-7 signaling, which led us to focus on its impact on IL-7 rather than TSLP function. In parallel we expressed the extra-cellular portion of IL7Rα

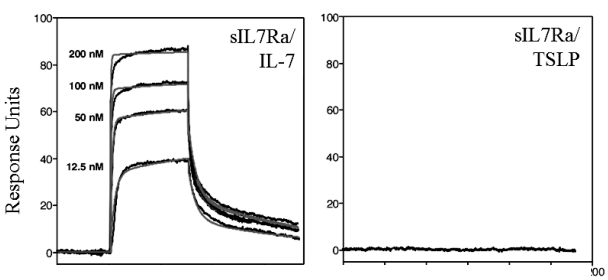

Figure 8: sIL7Rα binds IL-7 (left panel) but not TSLP (right panel). Sensograms illustrate binding curves determined by SPR.

to mimic the membrane bound isoform or a shed version of it. To our surprise we measured weaker IL-7 affinity for the IL7Rα extra cellular domain (K_d = 98 nM) than for the sIL7Rα (K_d = 6.3 nM). Whether this is due to structural changes of the binding site of sIL7Rα due to its unique 26 amino acid tail or not remains to be shown.

4.3.2 sIL7Rα potentiates IL-7 bioactivity

The connection between increased levels of sIL7Rα and increased MS risk, together with our findings that sIL7Rα binds IL-7 but not TSLP, rendered us with two possibilities:

1. *IL7R**C genotype → higher sIL7RA levels → diminished IL-7 signaling; hence IL-7 signaling prevents MS development; or
2. *IL7R**C genotype → higher sIL7RA levels → potentiated IL-7 signaling; hence IL-7 signaling can trigger MS development.

To test whether hypothesis 1 or 2 was the relevant one, we compared IL-7 consumption and signaling on a murine IL-7 dependent cell-line (2E8), human peripheral blood mononuclear cells (PBMC) and in *IL7*$^{-/-}$ mice. In all three experimental systems, co-injection of sIL7Rα + IL-7 led to reduced IL-7 consumption indicating competition between sIL7Rα and membrane bound IL7Rα. Furthermore, IL-7 induced survival of 2E8 cells and homeostatic proliferation of donated T-cells from a congenic strain in *IL7*$^{-/-}$ mice was increased suggesting potentiated IL-7 effect. We also found that EAE symptoms were worsened in C57/BL6 mice that received sIL7Rα+IL-7 compared to mice injected with IL-7 alone or PBS (Figure 9).

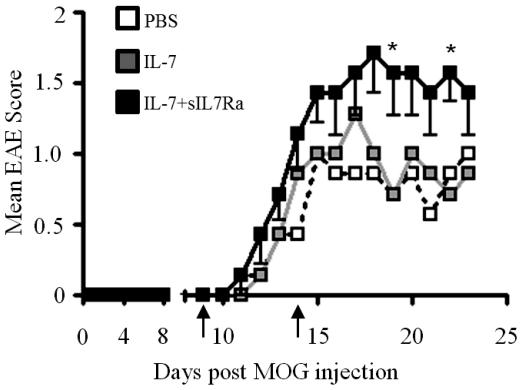

Figure 9: Injecting IL-7+sIL7Rα worsens EAE symptoms in mice compared to IL-7 alone or PBS

Apart from the quantitative differences, sIL7Rα also modulated the quality of the IL-7 signal in PBMC. Despite initial reduction in T-cell activation, over time sIL7Rα + IL-7 injection gave a more prolonged and potent stimulation than IL-7 alone. The IL-7 induced up regulation of the regulatory suppressor of cytokine signaling 1 (SOCS1) and CD95 molecules was (partially) inhibited in the presence of sIL7Rα. The overall picture from these experiments is that hypothesis 2 was accurate, and sIL7Rα provides an IL-7 depot that secures IL-7 availability over time and counters regulatory mechanisms induced by IL-7 alone. This model fits well with our current understanding of IL-7 as an immune stimulatory cytokine (*169*).

4.3.3 *IL7R* genotype influences sIL7RA and IL-7 levels

As expected, we saw increased expression (mRNA) and protein levels of sIL7Rα associate with *IL7R**C genotype (Figure 10). The effect on plasma IL7Rα was gene dose dependent in both MS patients and healthy controls. The genotype effect was of

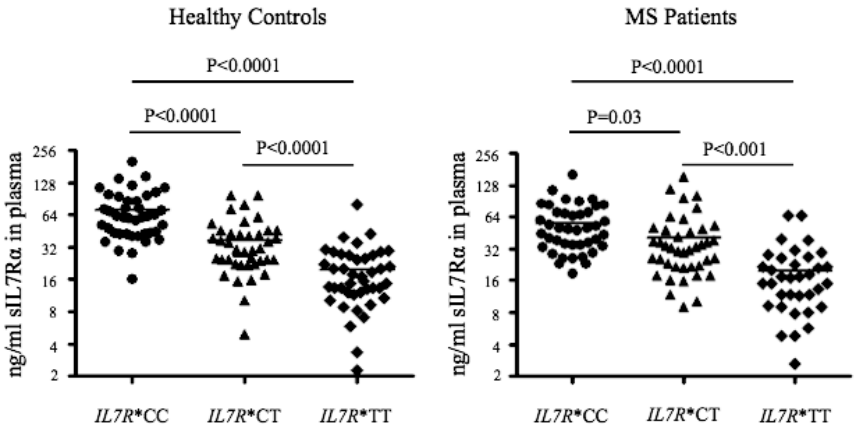

Figure 10: Plasma sIL7Rα concentration is determined by *IL7R* genotype in an allele-dose manner. The effect was seen in both MS patients and healthy controls.

similar size for mRNA and protein, indicating that alternative splicing is the main production method of sIL7Rα, as had been suggested before (*150*). We did not measure any sIL7Rα in CSF, either due to limitations in the detection level of our assay (ELISA), or because sIL7Rα is simply not crossing the BBB. Interestingly, we measured approximately twice as high plasma IL-7 levels in *IL7R**CC MS patients compared to *IL7R**TT MS patients or healthy controls (figure 11).

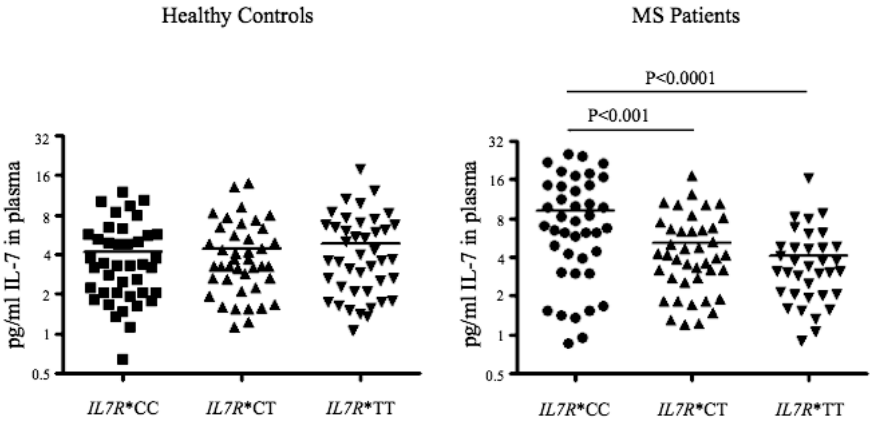

Figure 11: Plasma IL-7 levels vary with *IL7R* genotype in MS patients but not healthy controls

Why MS patients but not healthy controls carrying *IL7R**CC have increased plasma IL-7 is not clear. Either, there is an unknown genetic or environmental factor that together with an *IL7R**CC genotype increases systemic IL-7 in some individuals which then go on to develop MS. Conversely, MS itself might impact IL-7 levels by fluctuations in lymphocyte counts. Perhaps only patients with high enough sIL7Rα have the capacity to store excessive IL-7 in a sIL7Rα depot and hence maintain elevated plasma IL-7. It would be interesting to look for this genotype effect in patients with other diseases, as a first step towards understanding the difference based on MS biology.

4.3.4 Discussion

The *IL7R**C allele, responsible for increased soluble receptor levels and increased MS risk is, surprisingly, much more common than the MS protective allele (*IL7R**T; Figure 12). Several other autoimmune diseases are indirectly linked to IL7R*C (discussed in Section 2.1.5 of this thesis and (*109*)) which makes it even more surprising that there is evolutionary pressure towards it. A possible explanation could be that sIL7Rα actually helps the immune system to function properly. Since IL-7 is a limited resource *in vivo (125)*, the depot provided by sIL7Rα may help to prevent excessive consumption, thereby maximizing the immune stimulatory potential of IL-7. Another example of such a mechanism of IL-7 preservation is the altruistic down-regulation of a cell's membrane IL7Rα in response to IL-7 signaling (*110*). These tools of limiting excessive IL-7 consumption may be of great importance, since the production of IL-7 is stable and not thought to be actively regulated.

The bottom-line may be that the *IL7R**C allele increases the immune system's capacity to deal with infections by supplying high levels of sIL7Rα. This may come at the cost of a slight increase in various autoimmune diseases, but not to an extent threatening reproductive capacity. Inability to effectively fight infections on the other hand can be detrimental to survival in a much more direct way than post-adolescence onset of autoimmunity. Particularly in developing countries where limited access to medical facilities and poor hygienic standards are common, infectious diseases pose a great risk, especially in children and young adults (*170*). These circumstances may be the underlying factors why the most beneficial (*IL7R**TT) genotype from an MS perspective is virtually absent in African populations (Figure 12).

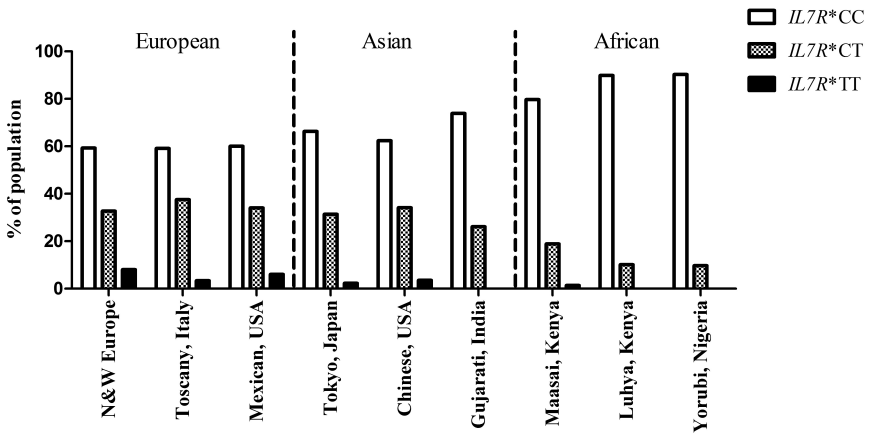

Figure 12: Homozygosity for the MS protective allele (*IL7R**TT) is rare across different ethnical ancestries. Public data obtained from phase 3 of the HapMap project (*171*).

It remains to be seen what the impact of these findings will be on the MS field. Targeting the IL-7 axis could certainly have its advantages, considering it would primarily impact non-T-regs. The increased understanding of sIL7Rα may additionally be relevant in other disease settings. RhIL-7 is currently being evaluated in numerous clinical trials, and perhaps its efficacy could be connected to sIL7Rα levels. A first step would be to genotype patients in these clinical trials for rs6897932, since there is such strong genetic correlation with endogenous sIL7Rα levels. Perhaps treatment efficacy can be improved, and negative regulation avoided with lower dose IL-7 + sIL7Rα compared to high dose IL-7 alone. Indeed, the most recently published results from a clinical trial of rhIL-7 as a supportive agent of immune reconstitution in bone-marrow transplant showed best results with intermediate levels (*172*). The highest dose gave less effective immune reconstitution suggesting regulatory mechanisms and excessive consumption may induce negative feedback mechanisms.

4.4 PAPER IV

4.4.1 IFNβ treatment leads to elevated serum IL-7

The initial objective of this study was to follow up and clarify the finding (*173*), and later dismissal (*174*) that high serum IL-7 levels before the start of IFNβ treatment predict good treatment response. We did not have the patient material to completely investigate a possible correlation between serum IL-7 and treatment outcome, however, we did notice elevated serum IL-7 across the board amongst IFNβ treated patients. Patients treated with natalizumab did not have increased IL-7 levels, suggesting the effect was not due to MS pathogenesis, but rather to IFNβ itself (Figure 13). Furthermore, switching treatment from IFNβ to natalizumab led to decreased serum IL-7 concentrations in the individual MS patients. The type of IFNβ compound injected did not influence IL-7 levels, but presence of neutralizing anti-drug antibodies (NAbs) did.

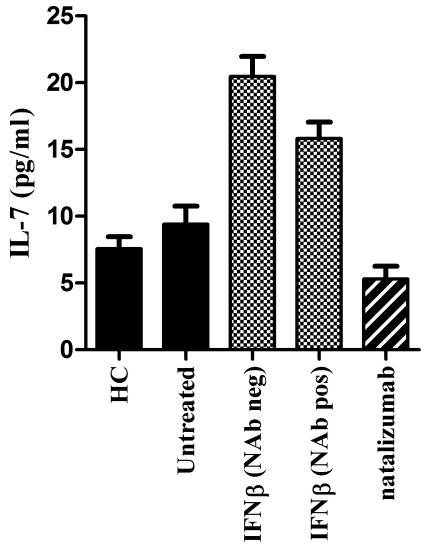

Figure 13: Serum IL-7 levels of healthy controls (HC) and MS patients on different treatments.

Since IL-7 is constitutively expressed, we hypothesized that the increased IL-7 levels were a product of decreased IL-7 consumption rather than increased IL-7 production. We focused on two parameters of critical importance for IL-7 consumption.

a) The number of IL-7 consuming cells in peripheral blood.

b) The rate of IL-7 consumption of individual cells.

4.4.2 IFNβ treatment leads to reduced IL-7 consumption

Addressing point a, we compared complete leukocyte, lymphocyte and monocyte counts in untreated, IFNβ treated and natalizumab treated MS patients. We saw a significant reduction in leukocyte and lymphocyte counts amongst IFNβ treated compared to untreated or natalizumab treated patients. The IFNβ effect was strongest in patients without NAbs. Natalizumab treatment on the other hand led to significantly increased leukocyte, lymphocyte and monocyte concentrations compared to untreated or IFNβ treated patients. This data suggests that IFNβ treatment, by inducing a mild

form of leuko- and lymphopenia, reduces the number of IL-7 consuming cells in circulation.

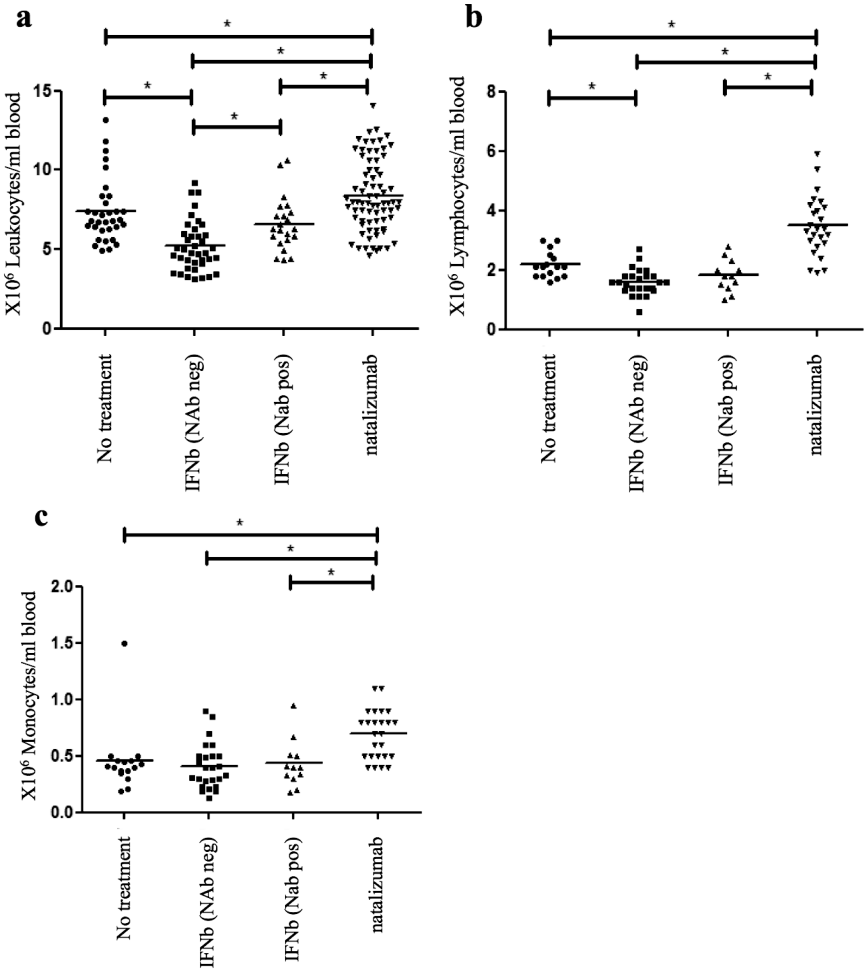

Figure 14: Peripheral blood leukocyte (a), lymphocyte (b) and monocyte (c) concentrations in MS patients. IFNβ treated patients are grouped by NAb status.

Although decreased IL-7 consumption due to reduced cell numbers could be the entire explanation, we wanted to look at IL7Rα expression in response to IFNβ to further elucidate the mechanism responsible for IL-7 elevation. *In vitro* we saw reduced surface expression of IL7Rα on T-cells and monocytes, as well as reduced IL-7 consumption in the presence of IFNβ. Furthermore, treatment naïve MS patients had significantly higher IL7Rα mRNA expression in CD3+ sorted cells before the start of treatment than ≥3 weeks after the first IFNβ injection. Plasma IL-7 was significantly increased at this time point, strengthening our *in vitro* observations of reduced IL-7 consumption in the presence of IFNβ.

4.4.3 Discussion

The results of these experiments present a "hen-or-the-egg problem": Does IFNβ signaling reduce IL7Rα expression, which drives up IL-7 levels? Or does IFNβ primarily reduce lymphocyte counts by other mechanisms leading to higher IL-7 levels due to reduced numbers of consuming cells, more IL-7 signaling on T-cells and hence IL7Rα down-regulation? Either way, increases in IL-7 are a potentially serious side-effect of IFNβ treatment.

This study, for the first time establishes a direct link between two of the currently most relevant and researched proteins in MS pathogenesis: IL-7 and IFNβ. This link between the only cytokine + receptor (*IL7* and *IL7R*) pair genetically associated with MS risk and the most widely used RRMS therapy lays the foundation for future research into the exact mechanisms involved as well as possible treatment modifications. Most researchers in the field of MS would agree, systemically elevated IL-7 levels are probably not beneficial for the disease modifying capabilities of IFNβ. Apart from the obvious involvement of IL-7 in various autoimmune processes, a recent report established that IL-7 induces up regulation of the α4 integrin utilized by T-cells to cross the BBB (*175*). Perhaps IFNβ treatment efficacy can be improved if IL-7 is targeted simultaneously.

5 SUMMARY OF FINDINGS

- *KIF1B* genotype may influence MS susceptibility
- *IL7R, IL2RA, SH2B3, CLEC16A* and *CD226* genotypes do not influence clinical course despite being associated with MS risk
- sIL7Rα binds IL-7 but not TSLP
- sIL7Rα potentiates IL-7 bioactivity *in vitro* and *in vivo*
- IL-7 but not sIL7Rα is present at low dose in the CSF
- Plasma IL-7 concentration depends on *IL7R* genotype in MS patients
- IFNβ leads to increased serum IL-7 levels by reducing consumption

6 CONCLUDING REMARKS

In the first years of this century, one of the (if not the) most ambitious scientific endeavor in history was finalized: the human genome project (*176, 177*). At that time, very few genetic associations with complex diseases were known, whereas today we know of over 1100 loci affecting more than 165 diseases and traits (*178*). The vast majority of these discoveries have happened over the last 5 years with the introduction of GWAS. MS genetics has covered a lot of ground since the start of the GWAS era going from 2 to more than 50 genetic loci associated to disease risk (*179*). A similar trend has been seen in several genetically complex diseases. Despite all this progress in understanding the genetics of disease, the clinical benefits for patients with MS as well as other complex diseases have been few to none. In a review article celebrating 10 years with the human genome, the current director of the National Human Genome Research Institute Dr. Eric Green speculates that the lack of clinical progress so far may not be entirely surprising (*180*). The authors reason, there is a lot of biological knowledge of disease processes left to acquire before we can really make the push into the clinics (Figure 15).

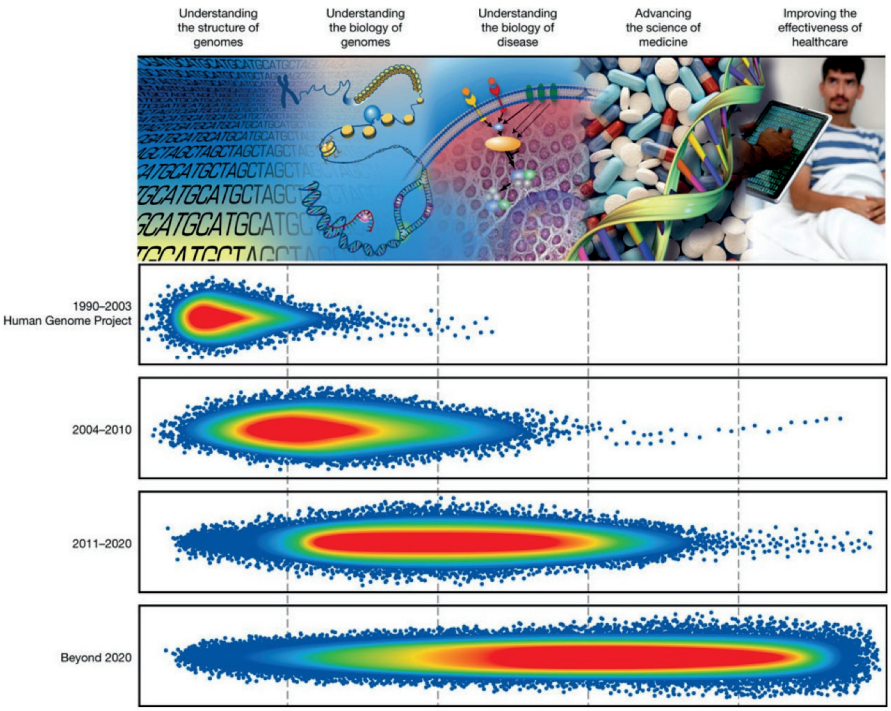

Figure 15: Schematic hypothetical heat map of individual discoveries (blue dots) in the path from genetic discoveries towards improved clinical care. Reprinted by permission from Macmillan Publishers Ltd: [Nature] (*180*), copyright (2011).

In MS, there is uncertainty about how much of the disease risk is determined by genetic versus environmental factors. Based on epidemiologic studies, the genetic contribution, termed heritability, is estimated to constitute 25-75% of the disease risk (*181*). The genetic associations to susceptibility determined so far do not add up to explain the entire hereditary portion of the disease. This is sometimes referred to as missing heritability. The size of this missing heritability is disputable since the size of the entire heritability of MS remains unclear. Probably gene-gene and gene-environment interactions as well as rare gene variants (which GWAS analyses do not detect) make up a substantial part of the missing heritability (*182*). These as well as individual genetic factors influencing disease need to be biologically understood in order to approach the goal of developing new treatments. Now is the time to use all the genetic knowledge we have acquired over the past five years and design clever functional studies. Only then can we make the progress Green *et al* expect and push the blue dots in Figure 15 towards their main purpose: to treat patients.

The scope of this thesis spans from determining genetic associations (paper I), via functional studies (paper III) to investigating clinical and treatment consequences (paper II and paper IV). Although I am very satisfied and proud of the work we have done over the last 4 years, there are many more experiments I wish I had already carried out, a few are listed below.

- For paper I, the reason behind the discrepancy between different reports needs to be thoroughly investigated. I think sub-division of the different patient cohorts by MS sub-phenotype (i.e. RRMS, SPMS, PPMS) is a good starting point. Furthermore an outside, unbiased critical statistical evaluation of how the p-values were calculated (assumptions of genetic homogeneity within cohorts, environmental exposures not accounted for etc.) would be very helpful to determine if there are false positives, false negatives, or simply differing approaches. Understanding the variation in results would not only be important from a historical perspective, but might help future GWAS attempts to be more fruitful and avoid pitfalls.

- It will be interesting to see whether the trend of paper II persists: susceptibility linked genes not influencing clinical parameters. On a different note, perhaps the EDSS scale is too prone to inter-physician variability in assessment to be useful in determining clinical influence of alleles with moderate influence on risk. I would like to see correlative studies between disease severity and a more objective outcome measure than EDSS e.g. MRI lesion load carried out.

- Paper III opens a lot of exciting possibilities for future experiments. First of all I would like to test IL-7 + sIL7Rα in an animal model of infection to see whether the *IL7R**C allele can help in this setting. If so, that could explain its high allele frequency across different populations and ethnicities despite its involvement in autoimmunity.

- Since IL-7 is in clinical trials as a mediator of immune reconstitution after disease or treatment induced lymphopenia, a quick experiment to test sIL7Rα's impact on IL-7 biology would be to genotype these patients for *IL7R*. Our results from paper III would predict that *IL7R**T carriers have a weaker response to recombinant human IL-7 since they have reduced sIL7Rα levels, and hence will experience a sharper peak and drop in IL-7 concentrations.

- sIL7Rα could potentially stabilize fluctuations in IL-7 levels as an indirect consequence of T-cell proliferation during an MS relapse. Since IL-7 is an important factor in the formation of immunologic memory, perhaps sIL7Rα ensures there is always a baseline IL-7 concentration capable of stimulating memory formation after a relapse. Particularly, CIS patients who do not develop MS would be interesting to genotype for *IL7R* and study more closely. Another way to approach the issue of memory would be to re-challenge an animal model (infect for infectious model or MOG for EAE model) and see if their responses differ based on injection with IL-7 alone or IL-7 + sIL7Rα (the hypothesis being that IL-7 + sIL7Rα injected animals reactivate their immunologic memory and hence have a stronger response than animals injected with IL-7 alone).

- To follow up paper IV I would like to go after the consequences of elevated IL-7 in IFNβ treated patients. Particularly, T-cell expression of α4 integrin, Treg counts and T-cell activation status would be interesting targets.

In conclusion, I have learned a lot over the past 4 years, and hopefully contributed a small piece to solving the MS-puzzle. In one of our first meetings, Dr. Crystal Mackall told me "This project might not lead to a cure for MS, but at least we will learn a lot about IL-7 biology". In retrospect, I think she was spot on.

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